Low CSF concentration of mitochondrial DNA in preclinical Alzheimer's disease.

Running Head: mtDNA in preclinical Alzheimer's disease CSF

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Abstract

Objective: To identify a novel biochemical marker that precedes clinical symptoms of Alzheimer's disease (AD).

Methods: Using quantitative PCR techniques, we measured circulating cell free mitochondrial DNA (mtDNA) in cerebrospinal fluid (CSF) from study participants, selected from a cohort of 282 subjects, that were classified, according to their concentrations of $A\beta_{1-42}$, t-tau and p-tau and by the presence or absence of dementia, in: asymptomatic subjects at risk of AD, symptomatic patients diagnosed with sporadic AD, pre-symptomatic subjects carrying pathogenic PSEN1 mutations and patients diagnosed with Fronto-temporal Lobar Degeneration (FTLD). We performed equivalent studies in a separate validation cohort of sporadic AD and FTLD patients. In addition, we measured mtDNA copy number in cultured cortical neurons from mutant Amyloid Precursor Protein/Presenilin1 (APP/PS1) transgenic mice.

Results. Asymptomatic patients at risk of AD, and symptomatic AD patients, but not FTLD patients, exhibit a significant decrease in circulating cell free mtDNA in the CSF. These observations were confirmed in the validation cohort. In addition, pre-symptomatic subjects carrying pathogenic PSEN1 gene mutations show low mtDNA content in CSF before the appearance of AD related biomarkers in CSF. Moreover, mtDNA content in CSF classifies with high sensitivity and specificity AD patients against either controls or patients with FTLD. Furthermore, cultured cortical neurons from APP/PS1 transgenic mice have less mtDNA copies, before the appearance of altered synaptic markers.

Interpretation: Low content of mtDNA in CSF may be a novel biomarker for the early detection of preclinical AD. These findings support the hypothesis that mtDNA depletion is a characteristic pathophysiological factor of neurodegeneration in AD.

Introduction

Recent evidence indicates that AD is preceded by a long preclinical phase with abnormal biochemical, structural and functional changes in the brain, suggesting that the pathophysiological process of neurodegeneration in AD starts well before the appearance of clinical signs of dementia. There are two biochemical markers, currently accepted to precede the appearance of clinical symptoms of dementia, that are widely used to define preclinical AD: 1) Brain accumulation of Amyloid β (Aβ) or low $A\beta_{1-42}$ content in CSF, and 2) Elevated CSF total tau (t-tau) and phosphorylated-tau (p-tau)[1,2]. Low concentrations of $A\beta_{1-42}$ in CSF are associated with AD[3,4] and correlate with accumulation of Aβ in the brain[5,6]. High CSF concentrations of t-tau and p-tau are associated with increased neocortical neurofibrillary tangles in AD affected brains at autopsy[7]. When used in combination, these two biomarkers support a good predictive value for negative AD diagnosis[4], but the diagnosis of AD is still probabilistic and requires the presence of clinical symptoms. The mechanistic relationship between the alteration of Aβ or tau biomarkers and the appearance of clinical signs of AD still remains elusive. Thus, brain amyloidosis appears to be necessary but not sufficient to produce the clinical signs of AD because post-mortem studies indicate that a considerable amount (10-30%) of people without dementia exhibit this neuropathological hallmark of AD [8,9]. Hence, the identification of new biomarkers that precede the appearance of clinical symptoms is crucial to advance in the knowledge of the pathophysiology of AD and for the development of therapeutic treatments.

CSF is in direct contact with brain parenchyma and it is considered to be the optimal fluid to identify possible alterations of brain metabolism[10]. However,

the relationship between brain and CSF levels of proteins in AD is not straightforward because the levels of CSF proteins such as tau correlate positively, while others such as Aβ correlate negatively with accumulation in brain. The reason why accumulation of Aβ in the brain is associated with low concentrations of $A\beta_{1-42}$ in the CSF is unclear, although it may be due to a decreased clearance of $A\beta_{1-42}$ from the brain to the CSF[11]. In contrast, a high concentration of t-tau in CSF is considered to reflect neuronal injury associated with accumulation of tau in the brain. However, other tauopathies in which there is high brain accumulation of tau in neurofibrillary tangles, do not exhibit a corresponding increase in CSF t-tau[12]. Puzzled with the opposite relationship between CSF and brain levels of Aβ and t-tau in AD, we initiated the search for new CSF biomarkers to gain further knowledge on the pathophysiological mechanisms of AD.

Converging evidence indicates that AD is associated with alterations in bioenergetics and mitochondrial function [13,14]. Neurons are highly dependent on aerobic energy provided by mitochondria, which contain several copies of their own DNA (mtDNA). Differently from genomic DNA, the covalently closed circular form of mtDNA renders it more resistant to degradation by nucleases, possibly present in the CSF. Because there are multiple copies of mtDNA per cell and mitochondria are distributed along axons, nerve terminals and dendritic arbors next to dendritic spines, we thought that the potential release of mtDNA to the extracellular space might be an index of mtDNA turnover in the brain. For this reason, we decided to explore whether it is possible to detect cell free mtDNA in the CSF using polymerase chain reaction techniques (PCR), which provide a relatively higher sensitivity and specificity than those offered by

currently available antibody-based protein detection procedures. We hypothesized that if cell free mtDNA could be detected in CSF, then mtDNA levels would reflect alterations in brain metabolism and might represent an early indicator of the neurodegenerative process in AD.

Methods

Subjects

The study subjects were selected from a cohort of 282 subjects recruited at the Alzheimer's disease and other cognitive disorders Unit of the Hospital Clinic of Barcelona. All subjects underwent clinical and neuropsychological assessment and lumbar puncture. Subjects were classified according to their concentrations of $A\beta_{1-42}$, total amount of microtubule-associated protein tau (t-tau), phosphorylated tau in threonine 181 (p-tau) and by the presence or absence of dementia (Table 1 and Supplementary Tables S1 and S2). Six groups were selected for the study: 1) AD, patients diagnosed with probable AD using NINCDS/ADRDA's criteria[15] and with the typical CSF biomarker profile characteristic of AD: low levels of $A\beta_{1-42}$ and elevated levels of t-tau and p-tau; 2) Low Aβ, asymptomatic subjects at risk to develop AD that do not have cognitive deficits and only have low CSF $A\beta_{1-42}$; 3) FTLD, symptomatic patients diagnosed with any of the syndromes incorporated within the Fronto-temporal Lobar Degeneration classification including progressive non-fluent aphasia, fronto-temporal dementia or semantic dementia, but with normal AD-related CSF biomarkers. 4) PSEN1, pre-symptomatic patients carrying a dominant PSEN1 mutation (M139T, K239N or I439S) with normal CSF biomarkers and normal cognition; 5) C1, age matched control group consisting of healthy subjects without cognitive deficits and normal CSF biomarkers to serve as control group for Low Aβ and AD groups; 6) C2, age-matched control group for PSEN1 mutation carriers composed by subjects of their family without clinical, genetic or biochemical AD related alterations. The following pathologic cut-off

values were used: $A\beta_{1-42}$ <500 pg/mL; t-tau >450 pg/mL; and p-tau >75 pg/mL. The subjects in all groups were finally balanced to avoid significant age differences between the groups . Only subjects <75 years of age were included in this study because of the lack of healthy controls without any altered biomarker above this age. Finally, samples with signs of blood contamination or from subjects with a CSF level of total protein above 0.7 mg/ml, were excluded from the study.

CSF samples

All CSF samples were obtained with informed consent at the Alzheimer's Disease and other cognitive disorders Unit of the Neurology Service, following the procedure approved by the ethics committee of the Hospital Clinic of Barcelona. The CSF samples were obtained by lumbar puncture between 9 a.m. and 12 p.m. A small initial volume of CSF was set aside to measure the presence of cells and perform other biochemical measurements and this was followed by collection of 10 ml of CSF. To prevent contamination of CSF with blood cells possibly acquired in the process of lumbar puncture, CSF samples were centrifuged immediately after collection at 2000 g for 10 min at 4ºC and stored within two hours after extraction in polypropylene tubes at −80 °C. Biomarkers $A\beta_{1-42}$, t-tau and p-tau were measured by enzyme-linked immunosorbent assay kits (Innogenetics, Ghent, Belgium). Subsequent PCR amplification with primers targeting the high copy nuclear gene 18S ribosomal RNA showed that the CSF samples used in our experiments do not contain nuclear DNA above the detection limit of 0.6 genomes per sample, ruling out

the possibility of CSF contamination with DNA from peripheral cells in the samples used for PCR studies.

Real time quantitative PCR

We could not find previous evidence showing the presence of circulating cell free mtDNA in human CSF. Hence, we first studied whether cell free circulating mtDNA can be detected in human CSF samples and next assessed its concentration by real time quantitative PCR with a standard calibration curve following MIQE guidelines[16]. Reactions were performed with at least ten replicates on Corbett Rotor-Gene 6000 (Corbett, Mortlake, NSW, 2137, Australia). Each 20 μl PCR reaction consisted of 1X SsoFast EvaGreen (Bio-Rad Laboratories, Hercules, CA 94547, USA), 300 nM forward/reverse primers and 6.2 μl of CSF sample. To detect mtDNA in the CSF, we designed PCR primers which amplify a region between bases 14418 and 14932 that encodes the mitochondrial genes ND6, tRNA-Glu and CYTB, according to the Cambridge reference sequence for human mitochondrial DNA (RefSeq: NC_012920.1). We choose only primers that are not associated with known single nucleotide polymorphisms. To confirm the specificity of the DNA amplified in the PCR reaction we designed three different combinations of primers targeting the same region of mtDNA (Fig 1A and B). Primer sequences 5'-3' are: F1- CCCCTGACCCCCATGCCTCA, R1-GCGGTGTGGTCGGGTGTGTT, F2- CTCACTCCTTGGCGCCTGCC, R2-GGCGGTTGAGGCGTCTGGTG. One primer combination, F1-R2, was designed to amplify a long 515 bp region (mtDNA-515). Two other primer pairs (F1-R1, F2-R2) were designed to amplify two short sequences (153 and 85 bp,

mtDNA-153 and mtDNA-85, respectively) that are within the long 515 bp region (Fig 1A). To verify the detection of mtDNA in CSF, we used another PCR technique based on hydrolysis probes with a primer combination targeting a different mitochondrial gene. For these studies, each 20ul PCR reaction consisted of 1X SsoFast Probes Supermix (Bio-Rad Laboratories, Hercules, CA 94547, USA), 100 nM forward/reverse primers, 125 nM Probe (5'-FAM-TGCCAGCCACCGCG-MGB-3') and 6.2 ul of CSF sample. The primers for this PCR reaction were designed to amplify a region between bases 805 and 927 of mtDNA genome that encodes the 12S mitochondrial ribosomal RNA gene. The sequences of this primer combination producing a 123 bp amplicon (mtDNA-123) are: Forward, 5'-CCACGGGAAACAGCAGTGAT-3'; Reverse, 5'CTATTGACTTGGGTTAATCGTGTGA-3'.

mtDNA may accumulate oxidative or structural damage and the longer the amplicon the higher the likelihood to have damaged bases that would prevent its amplification by the polymerase during the PCR reaction[17]. The PCR amplification of two different short sequences by the first two sets of primers (mtDNA-85 and mtDNA-153) was intended to confirm the specificity of the mtDNA target. The amplification of the longer sequence (mtDNA-515) was planned to assess the influence of mtDNA structural damage in the PCR reaction and as a further confirmation of mtDNA target specificity. The conditions for quantitative PCR were optimized using melting curve analysis to ensure the presence of a single amplification product. The characteristics of the amplified product were analyzed by both melting curve analysis and gel electrophoresis. Amplification was performed using the following cycle conditions: 95^0C, 2 min followed by 45 cycles at 95^0C, 5 s, and 65^0C, 10s, 20s

or 60s for mtDNA-85, mtDNA-153 and mtDNA-515 respectively. Quantification Cycle (Cq) measurements were set within the exponential range.

Quantification of cell free mtDNA in the CSF was performed with a standard calibration curve of human mtDNA purified from HEK293T cells. Human purified mtDNA was obtained from mitochondria isolated by subcellular fractionation and Percoll gradient. Mitochondria were isolated from $1x10^7$ HEK293T cells. Cells were washed, harvested in 2 ml of isolation buffer (20mM Hepes, 300mM Sucrose, 1mM EDTA, 1mM DTT, pH 7.4), and homogenized in a Glass/Teflon Potter-Elvehjem tissue grinder. The crude homogenate was centrifuged once at 1000g for 10 min to remove unbroken cells and nuclei. The crude mitochondria-containing supernatant was layered over a 23/15/10/3 % (v/v) discontinuous Percoll gradient in isolation buffer and centrifuged at 31.000g (Sorvall, FiberLite F21 rotor) for 5 mins at speed. The fraction containing mitochondria located between 15 and 23% Percoll layers was transferred to 1.5 ml microcentrifuge tubes and diluted 1:1 (v:v) in isolation buffer. After gentle mixing, mitochondria were centrifuged at 20000g for 20 min. The supernatant was discarded and DNA was extracted from the mitochondrial pellet using an alkaline lysis protocol (Wizard Plus Miniprep, Promega, Madison, WI, USA). The content of mtDNA in the mitochondrial DNA extract was assessed by different methods: a) by qPCR with three primer combinations against a standard curve of the respective amplicon cloned in pJET1.2 plasmid; b) by ddPCR. The concentration of mtDNA in CSF was calculated based on the assumption that one copy of mtDNA corresponds to 18.16 ag. Characterization studies indicated that mtDNA can be detected in human CSF samples in a range between 29-35 Cq, corresponding to a range of 2-300 fg/ml. Accordingly,

the concentration range of the standard calibration curve of mtDNA used in our experiments was 1-200 copies of mtDNA per reaction. PCR reaction efficiency was higher than 0.95 in all runs.

CSF ultrafiltration

Initially, we observed that direct mtDNA amplification in CSF samples produced PCR reactions with low efficiency (Supplementary Fig S1), indicating that CSF contains molecules that inhibit the detection of DNA by PCR, which compromise accurate quantification, as has been previously reported for other quantitative PCR assays in human body fluids. To achieve an accurate quantification of mtDNA, we performed several treatments of the CSF to remove PCR inhibitors and obtain PCR efficiencies equivalent to those obtained in our purified mtDNA standard. We found that ultrafiltration of the CSF completely removed PCR inhibitors yielding a PCR reaction efficiency equivalent to the observed in the mtDNA standard (Supplementary Fig S1) and indicating that the inhibitory substances present in CSF are soluble and of low molecular weight. CSF samples were ultrafiltrated using Amicon Ultra 0,5ml filters (Millipore, MA, USA) with 10000 MWCO. The samples were diluted 10 times the initial volume with PCR-grade water and concentrated back to the initial volume by centrifugation at 14000g for 10min. This ultrafiltration procedure was performed twice.

Droplet digital PCR

To validate results obtained with real time qPCR, we performed additional quantification of cell free circulating mtDNA in CSF samples from

some groups of the study cohort (Table 1 and Supplementary Table S2) by droplet digital PCR using Bio-Rad QX100 Digital Droplet PCR platform (Bio-Rad Laboratories, Hercules, CA 94547, USA). mtDNA was amplified directly in CSF samples with a FAM labeled probe and with the F1 and R1 primers that produce the mtDNA-153 amplicon used in the previous real time qPCR analyses. The reaction consisted of 1X ddPCR Mastermix, F1 and R1 primers at 900nM and a FAM labeled probe at 250nM. Probe sequence was 6-carboxyfluorescein (FAM)-5'-CGCTGTAGTATATCCAAAGACAACCATCATTCCCCC-3'-Black Hole Quencher-1 (BHQ-1). Droplet emulsion formation was performed by mixing 20ul reaction with 60ul droplet generation oil using a microfluidic droplet generation cartridge and QX100 Droplet Generator. The saturating PCR amplification was performed using C1000 Thermal Cycler with the following conditions. 95ºC 10min; 40 repeats of 94ºC 30sec 59ºC 1min; 98ºC 10min. PCR conditions were previously optimized using mtDNA purified from HEK293T cells. The presence or absence of amplification per droplet was evaluated using QX100 Droplet Reader (FAM channel) and analyzed using QuantaSoft protocol. The results were expressed in copies of mtDNA per μl of CSF.

Separate validation cohort: Subjects and qPCR methods

A separate validation cohort was collected to perform a replication study using a different qPCR method. The validation cohort was limited to sporadic AD and FTLD and consists of subjects recruited at the Neurology Service of the Hospital de Sant Pau of Barcelona and additional subjects recruited after the end of our first study at the Hospital Clinic of Barcelona (Table 1 and Supplementary Table S3). All subjects underwent clinical and

neuropsychological assessment and CSF samples were obtained by lumbar puncture with informed consent following the methods and rules approved by the ethics committee of the respective hospital of origin. Subjects were classified according their concentrations of CSF $A\beta_{1-42}$, t-tau, p-tau and by the presence or absence of dementia with the same inclusion criteria and pathological cut-off values used for the study cohort. Content of mtDNA was measured directly in CSF with an improved qPCR reaction procedure that circumvents the need of previous sample ultrafitration and with a new primer combination (mtDNA-73) which targets an mtDNA region different than the one targeted in the first study (Table 2). Reactions were performed with at least four replicates. The PCR reaction was in a final volume of 45.4 μl and consisted of 1X SsoFast EvaGreen (Bio-Rad Laboratories, Hercules, CA 94547, USA), 300 nM forward/reverse primers and 2 μl of CSF sample. The mtDNA-73 PCR primer pair amplifies a region between bases 13699 and 13771 that encodes the mitochondrial gene ND5, according to the Cambridge reference sequence for human mitochondrial DNA (RefSeq: NC_012920.1). The sequences of this primer combination producing an amplicon 73 bp long are: Forward, 5'-AAACGCCTGGCAGCCGGAAG-3'; Reverse, 5' GGAAGGGGGATGCGGGGGAA-3'. Amplification was performed using the following cycle conditions: 95^0C, 2 min followed by 45 cycles at 95^0C, 5 s, and 60^0C, 8s. Quantification of cell free mtDNA in the CSF was performed using an improved reference standard curve consisting of a known amount of copies of the plasmid pJET1.2 containing the mtDNA-73 amplicon. PCR reaction efficiency was higher than 0.95 in all runs.

Cortical Neuronal Culture

Primary cultures of cortical neurons were prepared from E17 embryo brain cortices from B6.Cg-Tg(APPswe,PSEN1dE9)85Dbo/Mmjax (APP/PS1) (Jackson Laboratories, Bar Harbor, ME, USA) mice of either sex. Cells were dissociated in the presence of trypsin and DNaseI and plated in poly-D-lysine (100 μg/ml)-coated dishes at a density of 2 x 10^5 cells/cm^2 in neurobasal medium supplemented with 2% B27, 0.1 mg/ml gentamicin, 0.5 mM glutaMAX. One third of the media volume was changed every 3-4 days. Cells were maintained at 37 ºC in a humidified incubator with 5% CO_2 and 95% air. The cultures were left in vitro undisturbed until the day (DIV) of the experiments. All procedures involving animals and their care were approved by the ethics committee of the University of Barcelona and they were conducted in accordance with guidelines that conform with national (Generalitat de Catalunya) and international laws and policies (Guide for the Care and Use of Laboratory Animals, National Academy Press, Washington, DC, 1996).

Genotyping of mice

Tail DNA was obtained following the protocol of The Jackson Laboratory (Bar Harbor, Maine, USA). 1 mm of tail was cut and incubated at 55 ºC in a buffer containing 50 mM KCl, 10 mM Tris.HCl pH 8.3, 2.5 mM $MgCl_2$•$6H_2O$, 0.45% (v/v) Nonidet P40, 0.45% (v/v) Tween 20 and 150 μg/ml Proteinase K (Gibco). After tissue digestion, the samples were incubated for 5 min at 95 ºC to inactivate Proteinase K and 2 μl of this sample were used for PCR analysis. Animals were genotyped by multiplex PCR using SSo Advanced Supermix (Bio-Rad Laboratories) with primers targeting the APP transgene and the IL-II gene

as a positive control. The primers were APP: Forward, 5'-CATAGCAACCGTGATTGTCATC-3'and Reverse, 5'-TGGATTCTCATATCCGTTCTGC-3'; IL-II: Forward, 5'-CTAGGCCACAGAATTGAAAGATCT -3'and Reverse, 5'-GTAGGTGGAAATTCTAGCATCATCC -3'. PCR conditions were: 2 min at 98 °C initial denaturation, followed by 45 cycles of 5s of denaturation at 98°C, 30s of primer annealing and extension at 60°C. Genotype was determined by melting curve analysis of PCR products.

Determination of mtDNA copy number in cultured cortical neurons

Total DNA was extracted by incubating cortical cultured neurons with SDS buffer (100 mM Tris-HCL, 10mM EDTA, 0.5% SDS, 20 μg/ml RNAse A, pH 8.0) for 1 h at 37º C in a extraction volume of approximately 1 μl per 1000 cells. Lysates were incubated with Proteinase K (100 μg/ml) for 1 h at 56º C followed by 10 mins at 95º C to inactivate the enzyme. Quantitative PCR was performed directly in extraction buffer to minimize the loss of mtDNA in column purification procedures [18]. We found that extraction buffer diluted 1:500 does not inhibit the efficiency of the PCR reaction. For mtDNA amplification, we used two primer combinations amplifying nested amplicons located within bases 214 to 912 of the 12S ribosomal RNA from of Mus musculus mitochondrion complete genome NC_005089.1: mtDNA-115 and mtDNA-699, producing amplicons of 115 and 699 base pairs, respectively. Each reaction contained 10 μl 2X SsoAdvanced Supermix (Bio-Rad Laboratories, Hercules, CA 94547, USA), 0.3 μM of each primer and 10 μl of 1:250 dilution of extracted sample for a final volume of 20 μl. The primer sequences are: mtDNA-115 (forward, 5'-

CTAGCCACACCCCCACGGGA-3' and reverse, 5'-CGTATGACCGCGGTGGCTGG-3'); mtDNA-699 (forward primer of mtDNA-115 and reverse, 5'-CGGGCGGTGTGTGCGTACTT-3'). PCR conditions were: 2 min at 95 °C initial denaturation, followed by 45 cycles of 5 s of denaturation at 95 °C and 12 or 60 s of primer annealing/extension at 60ºC or 65ºC for mtDNA-115 and mtDNA-699 respectively. For nuclear DNA (nDNA) quantification, we first amplified a sequence of 219 bp length of the nuclear multiple copy gene 18S ribosomal RNA. The primer sequences are: Forward, 5'-CGCGGTTCTATTTTGTTGGT-3' and Reverse, 5'-AGTCGGCATCGTTTATGGTC-3'. PCR conditions were: 2 min at 95 °C initial denaturation, followed by 45 cycles of 5 s of denaturation at 95 °C and 20 s of primer annealing/extension at 57ºC. All reactions were performed in triplicate. Quantification Cycle (Cq) measurements were set within the exponential range. Only reactions with efficiencies higher than 90% were considered. The absence of unspecific amplicons was confirmed by melting curve analysis and gel electrophoresis. Quantification of mtDNA and 18S ribosomal RNA nuclear DNA (nDNA) copies was obtained against a standard curve of the respective amplicons cloned in pJET1.2 plasmids. The results are expressed as mtDNA copies over 18S rRNA copies. In subsequent characterization experiments, we assessed the number of 18S rRNA copies present in the genome of cultured cortical neurons against a standard curve of two different single copy genes (IL-II and NP1) amplicons cloned in pJET1.2 plasmids. The results revealed that cultured cortical neurons have approximately 50 copies of 18S ribosomal RNA, indicating that these cells contain a range of 300-600 copies of mtDNA per cell.

Statistical Analysis

Results are expressed as mean ± SEM. The statistical significance of the differences was examined using Kruskal-Wallis test with Dunn's post hoc multiple comparisons, one-way ANOVA with Bonferroni post hoc multiple comparisons tests or with Two-tailed unpaired Student's t tests or Mann-Whitney U tests when required. Receiver operating characteristic curve and area under the curve analyses were performed with GraphPad Prism software.

Results

Detection of circulating cell free mtDNA in CSF

First we examined whether cell free mtDNA can be detected in CSF. Characterization studies showed that mtDNA can be detected by PCR amplification in a small volume of human CSF (10 μl) and that ultrafiltration of CSF removes inhibitory substances that prevent efficient PCR reactions and precise quantification (Supplementary Fig S1). To confirm the specificity of the amplified DNA, we used three different combinations of primers (Fig 1 and Table 2); one primer pair amplifying a long 515 bp region (mtDNA-515) and two other primer pairs, nested within this region, amplifying 85 bp (mtDNA-85) and 153bp (mtDNA-153) respectively (Fig 1A and 1B). PCR reactions were performed in CSF samples with each one of the three primer combinations. To rule out the possibility of non specific amplification, and verify the presence of only one amplicon, all PCR reactions were tested by melting curve analysis. The melting curve of the PCR reaction product from each primer combination was similar in all samples analyzed, with the exception of samples from subjects #1 and #51. The PCR products of the three primer combinations from these subjects, from study subject # 2 (Fig 1B) and from some other study subjects were sequenced and found to correctly match the corresponding targeted reference sequence NC_012920.1 of the human mitochondrial genome, with the exception of PCR products from subjects #1 and #51 which showed a G to A mutation in base number 14905 (Table 2). These results indicate that the products amplified by our primer combinations in CSF effectively correspond to mtDNA present in the CSF from study subjects and

not from external sources. PCR amplification with primers targeting the high copy nuclear gene for 18S ribosomal RNA showed that the CSF samples used in our experiments do not contain nuclear DNA above the detection limit of 0,6 genomes per sample, indicating that mtDNA we detected in CSF is cell free. To determine the concentration of mtDNA in CSF, we next performed real time quantitative PCR (qPCR) analyses in CSF samples, with an external standard consisting of a wide concentration range of purified human mtDNA isolated from HEK 293T cells. Results showed that mtDNA can be detected in human CSF in a range between 29-35 Cq, corresponding to concentrations of circulating mtDNA between 2-300 fg/ml. Equivalent results were obtained with a different qPCR analysis technique based on hydrolysis probes using a primer combination targeting a mtDNA region encoding the mitochondrial 12S ribosomal RNA gene (results not shown). No significant differences in CSF mtDNA levels were observed between males and females in any of the study groups (Fig S2).

CSF mtDNA levels in asymptomatic subjects at risk and sporadic AD patients

Next we analyzed the mtDNA content in CSF from subjects at the preclinical stage of AD, also named asymptomatic at risk, and from symptomatic sporadic AD patients (Table 1 and Supplementary Table S1). Amplification of the mtDNA-85 region revealed a marked reduction of cell free mtDNA content in CSF samples from both Low Aβ asymptomatic at risk subjects (LAβ=28±4 fg/ml, n=7) and symptomatic AD patients (AD=48±7 fg/ml, n=13), corresponding to a decrease of 85±2% and 74±3%, respectively, compared with the age matched control group 1 (C1=188±49 fg/ml, n=10). An

equivalent effect was observed when mtDNA concentration in CSF was assayed with the other two primer combinations. Thus, qPCR of mtDNA-153 showed a reduction of CSF mtDNA content of 96±1% and 84±4% in LAβ (6±1 fg/ml, n=7) and AD (23±6 fg/ml, n=13) groups, respectively, compared with the C1 group (142±44 fg/ml, n=10). Likewise, qPCR of the mtDNA-515 amplicon showed a reduction in CSF mtDNA content of 92±3% and 70±7% in LAβ (3±1 fg/ml, n=7) and AD (11±3 fg/ml, n=13) groups respectively, compared with the C1 control group (37±4 fg/ml, n=10) (Fig 1C). The AD patient group tended to have higher mtDNA content in CSF than the LAβ group in all three primer combinations, but this difference was not statistically significant. Overall, no significant differences were observed amongst groups in the percentage of decrease of mtDNA amplification between the longest amplicon (mtDNA-515) and the short amplicons (mtDNA-85 and mtDNA-153). Low mtDNA amplification could indicate either low mtDNA copy number or loss of DNA integrity. In the latter case, it is expected that the longer the amplicon the higher the reduction of PCR amplification, because long amplicons are more likely to accumulate damaged bases that would prevent its amplification by the polymerase. The equivalent reduction of mtDNA amplification observed in the present studies with amplicons of different length indicates that the decrease in mtDNA in LAβ and AD groups reflects a decrease in mtDNA copy number rather than a difference in mtDNA integrity. In summary, these results show that cell free mtDNA content in the CSF is reduced before the appearance of clinical symptoms, suggesting that low mtDNA is associated with the risk to develop AD.

CSF mtDNA levels in FTLD patients

To assess whether this decrease in the CSF content of mtDNA is also present in other non Alzheimer type dementias, we measured mtDNA concentration in CSF from symptomatic patients diagnosed with FTLD (Table 1 and Supplementary Table S1) [19]. Real time qPCR analysis revealed that mtDNA concentration in the CSF from these patients, as measured by the three different primer combinations (mtDNA-85= 132±35 fg/ml, mtDNA-153=234±160 fg/ml and mtDNA-515=77±27 fg/ml; n=15), was not significantly different from that found in C1 controls; but it was significantly higher than the observed in LAβ and AD groups (Fig 1C), indicating that AD and FTLD dementia do not share this decrease in cell free mtDNA concentration in CSF.

CSF mtDNA levels in preclinical subjects with PSEN1 mutations

To explore whether low mtDNA content in the CSF is present at the early preclinical stages of AD, we studied the CSF content of mtDNA in young pre-symptomatic subjects with a mutation causing AD before they reach a stage in which they have alterations in AD related biomarkers. We quantified cell free mtDNA concentration in CSF from a young group of cognitively normal subjects that do not show any alteration in Aβ and tau biomarkers but carry a pathogenic PSEN1 mutation (M139T, K239N or I439S) that will cause the full emergence of clinical AD symptoms on average at least one decade later (Table 1). Analysis of the CSF of these pre-symptomatic subjects also revealed a marked reduction in cell free mtDNA concentration in CSF, compared with the age matched control group. The magnitude of the reduction of mtDNA content in mutation carriers compared with controls was similar with the three different amplicons;

54±10% (PSEN1=27±6 fg/ml, n=6; C2=59±9 fg/ml, n=7), 68±11% (PSEN1=14±5 fg/ml, n=6; C2=43±12 fg/ml, n=7) and 66±11% (PSEN1=4±1 fg/ml, n=5; C2=13±3 fg/ml, n=7) for mtDNA-85, mtDNA-153 and mtDNA-515, respectively (Fig 1D). The results obtained in these pre-symptomatic PSEN1 subjects are consistent with those found in subjects at risk to develop AD (Fig 1C) and provide more evidence to suggest that reduced concentration of mtDNA in the CSF is present in early preclinical stages of AD. In contrast with the decrease of mtDNA content in CSF from patient groups, we found that in all primer combinations, mtDNA content in CSF is significantly higher ($p<0.01$) in the older C1 control group (mtDNA-85=188±49, mtDNA-153=142±44 and mtDNA-515=37±4 fg/ml; n=10) compared to the younger C2 control group (mtDNA-85=59±9, mtDNA-153=43±12 and mtDNA-515=13±3 fg/ml, n=10) indicating that mtDNA concentration in CSF increases with age, a process that is opposite to what we find in preclinical and asymptomatic at risk AD patients.

Receiver operating characteristic curve analysis of mtDNA CSF levels

To determine whether the amount of mtDNA in CSF efficiently discriminates between patients with sporadic AD and controls or FTLD we performed receiver operating characteristic curve (ROC) analysis of relative values of cell free mtDNA in CSF normalized to % control from all three (mtDNA-85, mtDNA-153, mtDNA-515) primer pairs combined (Fig 2A and 2B). The results showed that using a cut-off value of <50%, low CSF content of mtDNA distinguishes the diagnosis of sporadic AD from age matched controls with a sensitivity of 92%, specificity of 90%, respectively, and area under ROC curve of 0.992 (95% confidence interval= 0.967-1), significantly higher than

random ($p<0.001$). Likewise, with a cut-off value of <50%, the content of CSF mtDNA discriminates between AD and FTLD with a sensitivity of 92%, specificity of 87% and area under ROC curve of 0.98 (95% confidence interval= 0.957-1), significantly higher than random ($p<0.001$). Similar results were obtained when ROC analyses of mtDNA content between the different groups were performed with absolute data in fg/ml, either separate or combined, from each of the mtDNA amplicons (results not shown). These results provide further evidence to suggest that low mtDNA content in the CSF is specially related with AD.

Analysis of CSF mtDNA by ddPCR

Next we sought to determine whether the differences in CSF content of mtDNA found in our previous experiments could be validated with droplet digital PCR (ddPCR), a novel method that has made recently possible to directly quantify nucleic acid content at a single molecule resolution of target sequence. One advantage of this new PCR technique is that it does not require previous processing of the sample because the presence of inhibitory molecules does not significantly influence digital PCR quantification. Measurement of mtDNA copy number in CSF with ddPCR using mtDNA-153 amplicon primers confirmed that symptomatic AD patients have significantly lower number of mtDNA copies in CSF (AD=18.3±3.8 copies/μl, n=20) compared with the age matched control and FTLD groups (C1=41.8±7.9, n=9; FTLD=41.2±13.1, n=11, copies/μl), corresponding to a decrease of 56±9% of mtDNA in AD patients compared with the C1 group (Fig 3). These results are qualitatively equivalent to those found using real time qPCR and confirm our previous data showing that the

concentration of mtDNA in CSF in patients diagnosed with probable AD is different from those diagnosed with FTLD.

Analysis of CSF mtDNA in the separate validation cohort

To assess whether the findings of our first study are influenced by cohort characteristics or technical factors, we performed additional research in samples from a separate validation cohort of asymptomatic at risk of AD subjects and symptomatic AD and FTLD patients (Table 1 and Supplementary Table S3). Measurement of mtDNA copy number directly in CSF using an improved qPCR procedure with the mtDNA-73 primer combination confirmed that there is a significant reduction of cell free mtDNA content in CSF from Low Aβ asymptomatic at risk subjects (C-LAβ =44±8 copies/μl, n=5) and symptomatic AD patients (C-AD=67±9 copies/μl, n=17), corresponding to a decrease of 79±4 % and 69±4%, respectively, compared with the age matched control group (C-C= 214±47 copies/μl, n=19) (Fig 4A). No significant decrease in CSF mtDNA was observed in the C-FTLD group. These results are qualitatively equivalent to those obtained in the study cohort shown in Fig 1C. Moreover, in contrast with the study cohort, the validation cohort contains patients with two APOE ε4/ε4 alleles. In this cohort, analysis of the relationship between APOE genotype and CSF mtDNA levels showed that the APOE ε4 allele is associated with low CSF concentration of mtDNA (Fig 4B).

mtDNA copy number in cultured cortical neurons from APP/PS1 mice

Previous evidence has shown that there is a decrease in mtDNA copy number in postmortem brain from patients with AD[20], but in human brain tissue

it is not possible to determine when this effect starts and whether it is caused by the neurodegenerative process. To assess whether the decrease in the CSF concentration of mtDNA associated with AD occurs in the central nervous system before the appearance of neuronal damage, we measured the amount of mtDNA copies in cultured cortical neurons from transgenic mice expressing human APP/PS1 mutations. Neurons from these mice showed a significant decrease (~28%) of mtDNA copies per cell, as measured with two different primer combinations (Fig 5A). This decrease of mtDNA copy number occurs in cultured cortical neurons at 14 DIV well before the appearance of alterations in synaptic markers as measured by Synaptophysin and PSD95 protein levels (Fig 5B to D). These results are consistent with the interpretation that the decrease in cell free mtDNA content found in CSF of AD patients reflects a low mtDNA copy number per cell in central nervous system neurons and that low mtDNA content appears early in the neurodegenerative process of AD.

Discussion

Here we report that a decrease in the CSF concentration of circulating cell free mtDNA precedes the appearance of core AD biomarkers in the CSF of pre-symptomatic subjects who will develop AD. The reduction of mtDNA content found in the CSF of *PSEN1* mutation carriers occurs at least one decade before these patients are expected to manifest clinical signs of dementia and appears before any change in the CSF levels of $A\beta_{1-42}$, the first pathological sign known to be altered in the preclinical stage of AD. This suggests that the pathophysiological process of AD would start earlier than previously thought and that the content of mtDNA in the CSF, like some neuroimaging procedures, may be one of the earliest biomarkers of the long neurodegenerative course of the disease. Likewise to familial AD, we find that low mtDNA concentration in the CSF is also present in sporadic AD. Thus, depletion of CSF mtDNA occurs in patients diagnosed with probable sporadic AD as well as in asymptomatic subjects at risk to develop AD, before alterations in t-tau and p-tau. The finding that low mtDNA concentration is a common feature in the CSF of both pre-symptomatic mutation carriers who will develop familial AD at an early age and asymptomatic subjects at risk to develop sporadic AD, suggests that mtDNA content in the CSF may help to detect early the preclinical stage of the disease.

Moreover, we find that mtDNA content in CSF can distinguish patients diagnosed with AD from either control or patients with FTLD. Patients in each of these groups, as well as the corresponding control subjects, were carefully selected, to the detriment of the number of patients per group, according to the presence or absence of clinical, genetic and biomarker criteria to obtain the

highest possible homogeneity within experimental groups and avoiding any biomarker overlap amongst groups. The high efficiency and specificity that mtDNA content in CSF exhibits for the differential diagnosis of AD and FTLD, together with the observation that low mtDNA in CSF occurs in presymptomatic pathogenic PSEN1 mutation carriers well before the appearance of any clinical or biomarker signs, suggests that reduction of mtDNA content in CSF is a particular pathophysiological factor of AD. Moreover, the present results imply that the pathophysiology underlying AD type dementia is different from FTLD, which otherwise are often difficult to distinguish in terms of their clinical presentation. At present, we do not know if the decrease of mtDNA in CSF occurs also in other neurodegenerative disorders in addition to AD. As the recruitment of more voluntary controls and subjects at risk for other neurodegenerative disorders progresses, future studies will determine whether the decrease in mtDNA occurs in other neurodegenerative diseases. However, the finding that low content of CSF mtDNA occurs both in familial and sporadic forms of AD before the appearance of clinical symptoms, but not in symptomatic patients with dementia of the FTLD type, supports the interpretation that altered mtDNA replication or degradation is a key pathophysiological mechanism in the early stage of AD.

The discovery that low CSF content of cell free circulating mtDNA is associated with sporadic AD and is already present in asymptomatic subjects at risk of AD was replicated in a separate validation cohort, which included samples obtained in another hospital. Moreover, in contrast with the study cohort, the validation cohort contains samples from patients with the APOE $\varepsilon4/\varepsilon4$ alleles, the main genetic risk factor for late onset AD. Interestingly, the

analysis of the relationship between APOE genotype and CSF mtDNA showed that the APOE ε4 allele is associated with low CSF concentration of mtDNA. This might be because all subjects with the APOE ε4/ε4 genotype fell in the AD group; we could not find subjects with this genotype in the other groups. However, the relationship between APOE ε4 allele and mtDNA decline is consistent with the interpretation that CSF mtDNA concentration is an early indicator of the neurodegenerative process in AD. Although more long term studies in different laboratories with neuropathologically confirmed cases are necessary to validate whether mtDNA content in CSF is an early biomarker of AD, the present results suggest that mtDNA depletion is a fundamental biological process in the disease.

It is well established that patients with AD exhibit significant neuronal death. Hence, the finding that preclinical subjects at risk of AD have lower mtDNA concentration in CSF appears to be contrary to what should be expected if the origin of mtDNA in the CSF is damaged brain neurons or broken cells from choroid plexus or periventricular zones. However, the present results support the interpretation that the amount of circulating mtDNA in the CSF, in the absence of cell damage, is related with the amount of mtDNA per cell. Thus, the low mtDNA content found in both asymptomatic subjects at risk of AD and pre-symptomatic mutation carriers that have not reached the stage of structural brain damage, is consistent with the hypothesis that brain cells of all these subjects have less mtDNA before significant signs of neurodegeneration. Actually, the results reported here are in agreement with recent evidence showing that postmortem brains from patients with AD exhibit low mtDNA copy number and impaired mitochondrial biogenesis [20,21], which supports the

interpretation that cell free circulating mtDNA content in the CSF is a reflection of the amount of mtDNA per cell in the brain. Moreover, the finding that depletion of mtDNA precedes alterations in synaptic markers in cultured cortical neurons from APP/PS1 mice (Fig 5) is consistent with the notion that changes in mtDNA content occur early in the neurodegenerative process. Furthermore, the finding that mtDNA content in CSF increases with age (Fig 1), which is opposite to what we find in preclinical AD, provides support to the notion that low mtDNA content in CSF is an index of a particular pathophysiological factor related with AD that occurs before neuronal cell damage.

More studies are needed in both animal models and human therapeutic clinical assays to determine whether the reduction in cell free mtDNA content in CSF reported here mirrors an etiological factor of the neurodegenerative process in AD, and whether CSF mtDNA concentration may change with disease-modifying treatments. Longitudinal studies in human therapeutic trials to determine whether new disease –modifying treatments are capable of returning AD related protein biomarkers to normal levels are hampered by the technical difficulties associated with current antibody-based protein detection procedures. One important advantage of measuring mtDNA as an index of preclinical AD is that mtDNA can be detected by normal qPCR or by droplet digital PCR, facilitating the assessment of larger cohorts in longitudinal studies with higher sensitivity and specificity. CSF contains factors that inhibit the PCR reaction and these factors can be removed by ultrafiltration and not by treatment with proteinase K, indicating that the inhibitory molecules are not proteins. However, we found that PCR detection of mtDNA in CSF either after ultrafitration or directly in a very small non-inhibitory volume of CSF yields

qualitatively equivalent results. Direct measurement of CSF mtDNA by real time qPCR or by ddPCR may enable more laboratories to investigate the role of mtDNA in AD progression and widen the possibility to identify new treatments.

We speculate that if the magnitude of the decrease of cell free mtDNA that we find in CSF directly reflects what happens in neurons it would likely be a causal factor for neurodegeneration by cellular energy deprivation, because neurons are highly dependent on aerobic energy provided by mitochondria. The finding that low mtDNA content occurs in the CSF from subjects at risk of both sporadic and familial forms of AD, but not in subjects with non-AD dementia, provides strong evidence to hypothesize that regulation of mtDNA copy number is a common mechanism onto which different pathways causing AD converge. In neurons, many different mechanisms that regulate mitochondrial function can cause depletion of mtDNA, and those can be either intrinsic to mitochondrial function, i.e oxidative stress, or extrinsic such as Aβ accumulation. Recent evidence showing that proteins that cause AD when mutated, such as PSEN1 and PSEN2 complexes and APP, are associated with mitochondrial membranes[22,23] and may regulate mitochondrial function is consistent with this hypothesis. The heuristic hypothesis that we propose here postulating that low mtDNA content in CSF is a pathophysiological biomarker of AD would integrate with the more general unifying hypothesis which proposes that the pathophysiology of AD is caused by bioenergetic decline due to mitochondrial dysfunction[13,14,24]. More research stemming from the hypothesis that low mtDNA concentration in CSF is associated with risk of AD may help the development therapeutic treatments and also to increase the knowledge of the pathophysiological mechanisms causing dementia in AD.

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Table 1. [a] Subjects for the study cohort were selected from a cohort of 282 subjects. [b] Available CSF samples from the study cohort were used to determine CSF mtDNA copy number. Additional subjects were incorporated in the AD group. [c] Groups of the validation cohort (C-) included 25 subjects recruited at the Neurology Service of the Hospital de Sant Pau of Barcelona and 25 subjects recruited independently from the first study at the Hospital Clinic of Barcelona. [d] CSF samples were classified according to the concentrations of $A\beta_{1-42}$, t-tau and p-tau and by the presence or absence of dementia in the subjects. C1: age matched control group consisting of healthy subjects without cognitive deficits and normal CSF biomarkers. LAβ: asymptomatic subjects at risk to develop AD that do not have cognitive deficits and only have low CSF $A\beta_{1-42}$. AD: patients diagnosed with probable sporadic Alzheimer's disease with low levels of $A\beta_{1-42}$ and elevated levels of t-tau and p-tau. FTLD: patients diagnosed with Frontotemporal Lobar Degeneration and with normal AD-related CSF biomarkers. C2: age matched control group, which includes subjects without clinical, genetic or biochemical AD related alterations as a control for PSEN1 group. PSEN1: pre-symptomatic patients carrying a dominant pathogenic Presenilin 1 mutation (M139T, K239N or I439S) with normal CSF biomarkers and normal cognition. N=number of subjects per group. ** Significantly different from the corresponding control group (Oneway ANOVA with Bonferroni post-hoc comparisons, $p<0.01$).

Table 2. PCR reactions were performed in human CSF samples with each one of four primer combinations as described in Methods section. Each reaction was tested by melting curve analysis to rule out the possibility of non specific amplification and verify the presence of only one amplicon. The melting curve of the amplicons from each primer combination was the same in all samples, with the exception of samples from subjects #1 and #51 in primer combination F1-R2 and subjects #80 and #93 in primer combination F3-R3. The PCR products of the four primer combinations obtained from these and other subjects (including the representative CSF amplicons from study subject #2. shown in Fig. 1B) were sequenced by dsDNA sequencing to verify that their sequence corresponds to human mtDNA. The sequence of the amplicons obtained in the first three primer combinations correctly matched the corresponding targeted reference sequence NC_012920.1 of the human mitochondrial genome in all samples analyzed, with the exception of the amplicons mtDNA-515 and mtDNA-85 from subjects #1 and #51 that share a G to A mutation in base number 14905. In all samples analyzed, the amplicon of mtDNA-73 differed in one base (A to T) from the reference mtDNA sequence.

Figure 1. Reduction of mitochondrial DNA concentration in cerebrospinal fluid from preclinical Alzheimer's disease subjects. Real time quantitative PCR was performed to measure mtDNA content in CSF samples with primers targeting a region between bases 14418 and 14932 of human mtDNA. A) Three different combinations of primers, two of them nested, were used to confirm specificity of mtDNA in the CSF by PCR amplification. Two sets of PCR primers (F1-R1, F2-R2) were designed to amplify two short sequences (153 bp and 85 bp, mtDNA-153 and mtDNA-85, respectively) located within a close distance of human mtDNA at the extremes of a longer, 515 bp (mtDNA-515) sequence, that could be amplified also by the combination of the forward and reverse primers of the first and second primer set respectively (F1-R2). B) Representative image of an agarose gel showing DNA size marker (M), mtDNA-153 (Lane 1), mtDNA-85 (Lane 2) and mtDNA-515 (Lane 3) amplicons obtained with the three different primer pairs in a CSF sample from study subject number 2. C,D) Quantification of mtDNA content by real time PCR in CSF from asymptomatic subjects at risk, AD and FTLD patients (C) and pre-symptomatic PSEN1 mutation carriers (D). Values are mean ± SEM of mtDNA concentration in CSF expressed in fg/ml. The number above error bars represents the number of subjects per group. *, $p<0.05$; **, $p<0.01$, significantly different from the corresponding age matched control group (Kruskal-Wallis test with Dunn's post-hoc comparisons). #, $p<0.05$, significantly different from the corresponding age matched control group (Mann-Whitney U test, two tailed).

Figure 2. Receiver operating characteristic curve analysis. Sensitivity and specificity of relative values of cell free mtDNA in CSF shown in Fig 1C, from all three (mtDNA-85, mtDNA.153, mtDNA-515) primer pairs combined and normalized to % control, to correctly categorize patients with a diagnosis of: A) Cognitively normal age matched controls, C1 (n=10) versus Probable Alzheimer's disease, AD (n=13); area under the curve = 0.992, $p<0.001$; B) Frontotemporal Lobar Degeneration with normal AD-related biomarkers, FTLD (n=15) versus Probable Alzheimer's disease, AD (n=13); area under the curve= 0.982, $p<0.001$.

Figure 3. Quantification of cell-free mtDNA copies in CSF samples by ddPCR. mtDNA copy number was measured directly in CSF samples by droplet digital PCR using Bio-Rad QX100 Digital Droplet PCR platform. The mtDNA-153 amplicon was obtained with F1 and R1 primers and a FAM labeled probe. Results were analyzed using QuantaSoft program. Values are expressed as mean ±SEM of number of mtDNA copies per μl of CSF. C1, Age matched control group. AD, patients diagnosed with probable sporadic Alzheimer's disease with low $A\beta_{1-42}$ and high t-tau and p-tau CSF levels. FTLD, patients diagnosed with Frontotemporal Lobar Degeneration and with normal AD-related CSF biomarkers. The number above error bars represents the number of subjects per group. **, $p<0.01$, significantly different from C1 (Kruskal-Wallis test with Dunn's post-hoc comparisons).

Figure 4. Analysis of mtDNA content and relationship with APOE genotype in CSF samples from a separate validation cohort of sporadic AD. CSF was assayed directly in real time quantitative PCR reaction with the mtDNA-73 primer combination targeting a region between bases 13699-13771 of human mtDNA. (A) mtDNA concentration expressed in number of copies/μl in CSF from age matched controls (C-C), asymptomatic subjects at risk (C-LAβ) and symptomatic patients diagnosed with AD (C-AD) or FTLD patients (C-FTLD) of the validation cohort. (B) Analysis of the relationship between APOE genotype and mtDNA content in CSF. Values are mean ± SEM of mtDNA concentration in CSF expressed in copies/μl. The number above error bars represents the number of subjects per group. *, $p<0.05$; **, $p<0.01$, significantly different from control group (Kruskal-Wallis test with Dunn's post-hoc comparisons).

Figure 5. Reduction of mtDNA copy number occurs before alterations in synaptic markers in cultured cortical neurons from APP/PS1 mice. mtDNA copy number and synaptic proteins were measured in cultured cortical neurons from wildtype (WT) and APP/PS1 mice. A) mtDNA was measured in cultured cortical neurons at 14 DIV. Number of mtDNA and 18S rRNA copies were determined against reference plasmids containing the amplified sequences. For mtDNA, two different primer combinations were used: mtDNA-115 and mtDNA-699, producing amplicons of 115 and 699 base pairs, respectively. Values are mean ± SEM of n=number of animals, of mtDNA copies/copy of 18S rRNA. WT (n=8), APP/PS1 (n=16). *, $p<0.05$; **, $p<0.01$, significantly different from the corresponding control (Student's unpaired t-test, two tailed). B) Representative western blots showing PSD95 and Synaptophysin (Syn) protein levels in

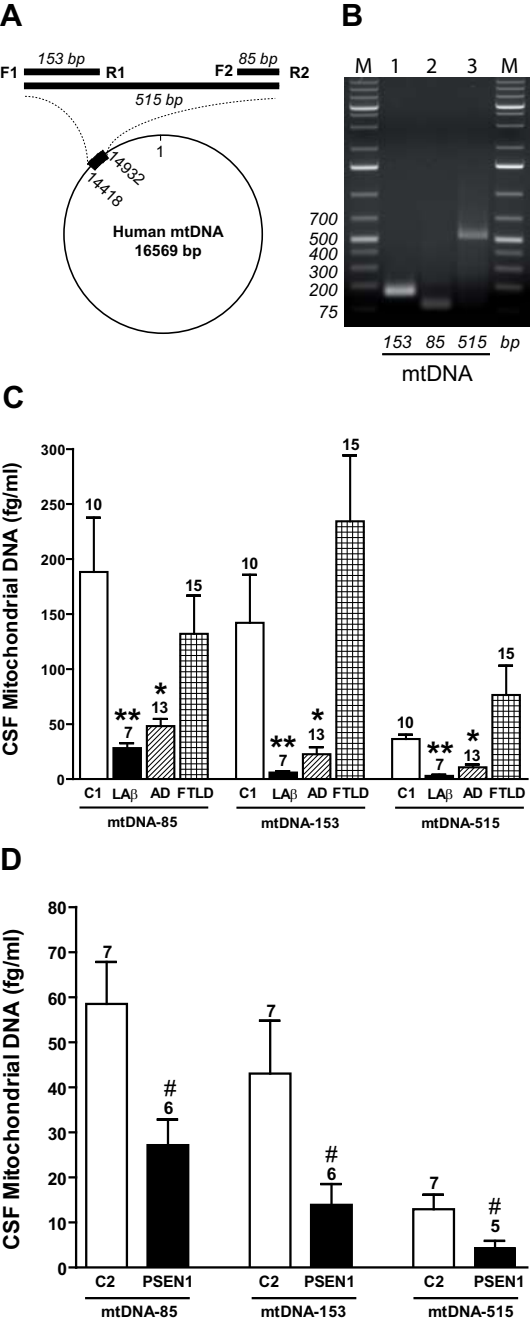
cultured cortical neurons lysates from WT and APP/PS1 mice at 14 and 21 DIV. Proteins were separated by SDS-PAGE and transferred to PVDF membranes. C-D) Quantitative analysis PSD95 and Synaptophysin protein levels. The densitometric values of the bands representing PSD95 (C) or Synaptophysin (D) immunoreactivity were normalized to the values of the corresponding actin band. Values are mean ± SEM for PSD95 (WT n=11 and 11; APP/PS1 n=9 and10, for 14 and 21 DIV, respectively) and for Synaptophysin (WT n=12 and 10; APP/PS1 n=9 and 10, for 14 and 21 DIV, respectively). **, $p<0.01$, significantly different from the corresponding control (Student's unpaired t-test, two tailed).

Table 1. Age and biomarker characteristics of subject groups in study and validation cohorts

GROUP[d]	N	Age	Aβ [pg/ml]	t-tau [pg/ml]	p-tau-T181 [pg/ml]
Study Cohort[a]: Groups analyzed by Real Time qPCR					
C1	10	62±1	883±73	226±15	52±3
LAβ	7	67±3	364±32**	162±22	40±6
AD	13	64±2	302±18**	883±125**	119±13**
FTLD	15	61±2	718±40	268±27	47±3
C2	7	38±3	690±25	261±17	53±1
PSEN1	6	35±3	908±163	246±19	51±3
Study Cohort[b]: Groups analyzed by ddPCR					
C1	9	62±1	885±81	227±17	52±3
AD	20	65±1	328±14**	897±95**	123±10**
FTLD	11	60±3	772±43	313±38	55±4
Validation Cohort[c]: Groups analyzed by Real Time qPCR					
C-C1	19	61±1	808±41	207±10	49±3
C-LAβ	5	67±4	419±37**	197±39	38±6
C-AD	17	66±1	321±21**	833±80**	95±5**
C-FTLD	9	60±2	734±37	234±24	42±2

Table 2. Sequences of Human mtDNA amplified by Real Time qPCR

Primer Combination	PCR Amplicon	Human mtDNA Region Ref Seq: NC_012920.1	Sequence 5'-3'
F2-R2	mtDNA-85 bp	14848-14932	CTCACTCCTTGGCGCCTGCCTGATCCTCCAAATCACCACAG GACTATTCCTAGCCATG*CACTACTCACCAGACGCCTCAACC GCC
F1-R1	mtDNA-153 bp	14418-14570	CCCCTGACCCCCATGCCTCAGGATACTCCTCAATAGCCATC GCTGTAGTATATCCAAAGACAACCATCATTCCCCCTAAATAA ATTAAAAAAAACTATTAAACCCATATAACCTCCCCCAAAATTCA GAATAATAACACACCCGACCACACCGC
F1-R2	mtDNA-515 bp	14418-14932	CCCCTGACCCCCATGCCTCAGGATACTCCTCAATAGCCATC GCTGTAGTATATCCAAAGACAACCATCATTCCCCCTAAATAA ATTAAAAAAAACTATTAAACCCATATAACCTCCCCCAAAATTCA GAATAATAACACACCCGACCACACCGCTAACAATCAATACTA AACCCCCATAAATAGGAGAAGGCTTAGAAGAAAACCCCACA AACCCCATTACTAAACCCACACTCAACAGAAACAAAGCATAC ATCATTATTCTCGCACGGACTACAACCACGACCAATGATATG AAAAACCATCGTTGTATTTCAACTACAAGAACACCAATGACC CCAATACGCAAAACTAACCCCCTAATAAAATTAATTAACCAC TCATTCATCGACCTCCCCACCCCATCCAACATCTCCGCATG ATGAAACTTCGGCTCACTCCTTGGCGCCTGCCTGATCCTCC AAATCACCACAGGACTATTCCTAGCCATG*CACTACTCACCA GACGCCTCAACCGCC *G-A in subjects #1 and #51
F3-R3	mtDNA-73 bp	13699-13771	AAACGCCTGGCAGCCGGAAGCCT[1]A[2]TTCGCAGGATTTCTCA TTACTAACAACATTTCCCCCGCATCCCCCTTCC [1]Deletion in subjects #80 and #93 [2]A-T in all samples


A
153 bp
F1
R1
85 bp
F2
R2
515 bp
1
14932
14418
Human mtDNA
16569 bp
B
M
1
2
3
M
700
500
400
300
200
75
153
85
515
bp
mtDNA
C
CSF Mitochondrial DNA (fg/ml)
300
250
200
150
100
50
0
10
7
13
15
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C1
LAβ
AD
FTLD
mtDNA-85
mtDNA-153
mtDNA-515
D
CSF Mitochondrial DNA (fg/ml)
80
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60
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20
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7
6
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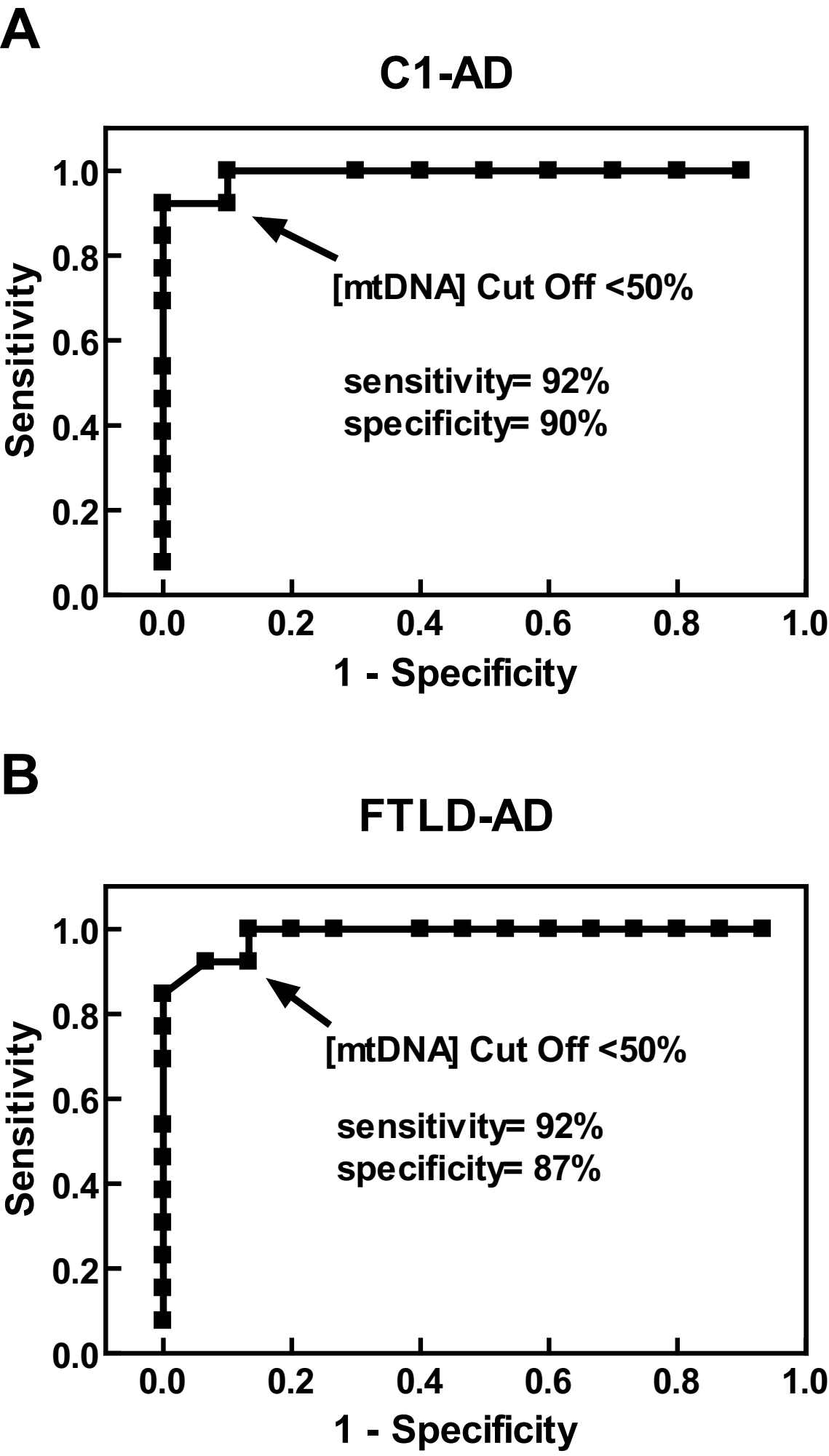
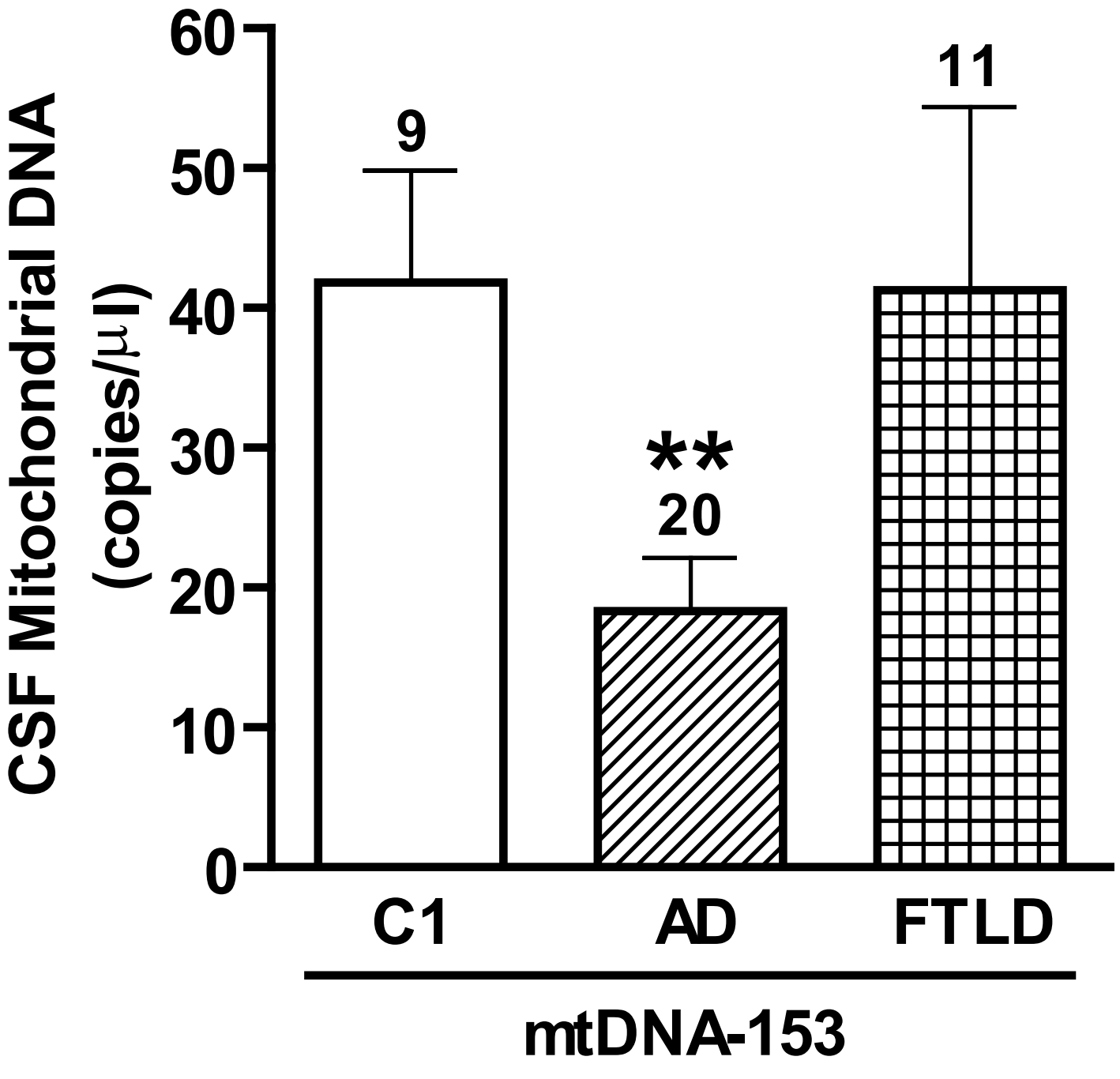
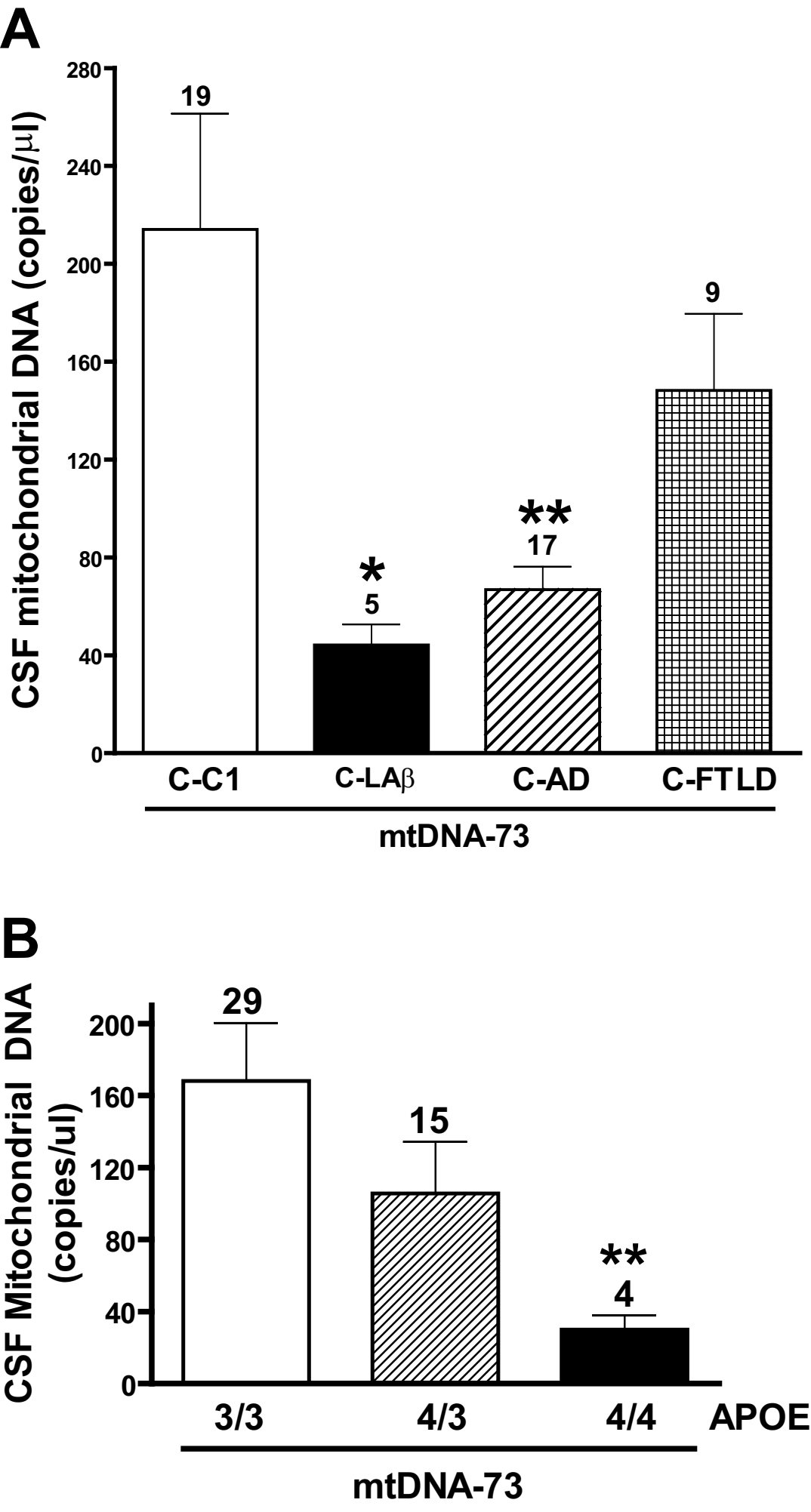
C2
PSEN1
mtDNA-85
mtDNA-153
mtDNA-515


A
C1-AD
Sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
[mtDNA] Cut Off <50%
sensitivity= 92%
specificity= 90%
0.0
0.2
0.4
0.6
0.8
1.0
1 - Specificity
B
FTLD-AD
Sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
[mtDNA] Cut Off <50%
sensitivity= 92%
specificity= 87%
0.0
0.2
0.4
0.6
0.8
1.0
1 - Specificity


CSF Mitochondrial DNA
(copies/μl)
60
50
40
30
20
10
0
9
**
20
11
C1
AD
FTLD
mtDNA-153


A
CSF mitochondrial DNA (copies/μl)
280
240
200
160
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80
40
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19
*
5
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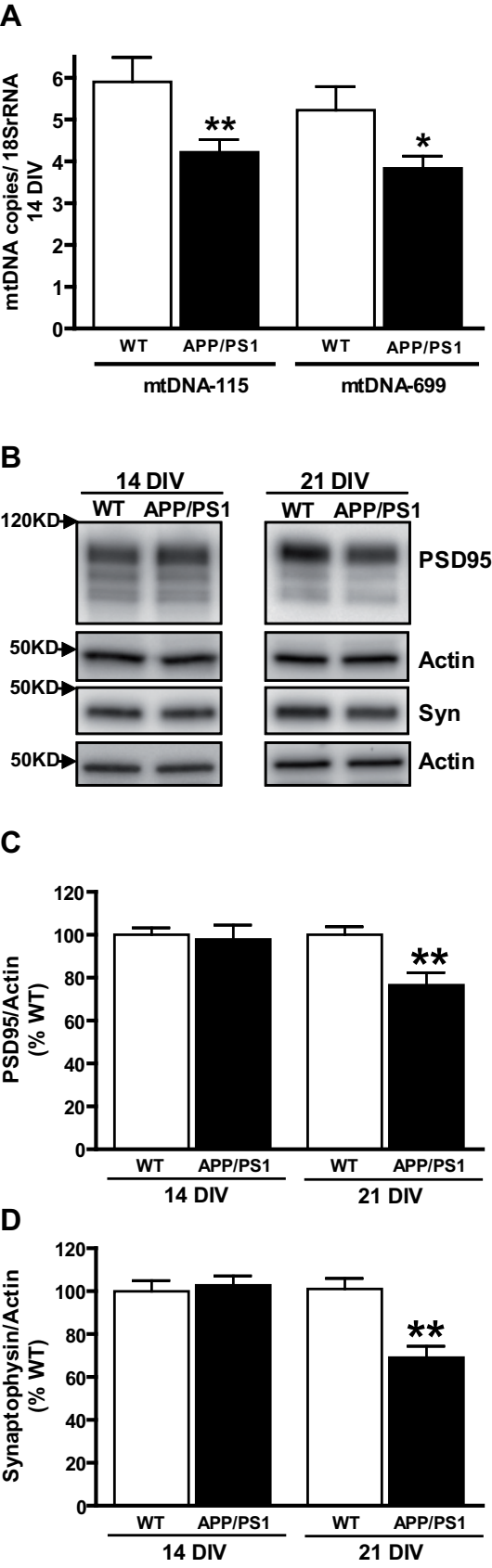
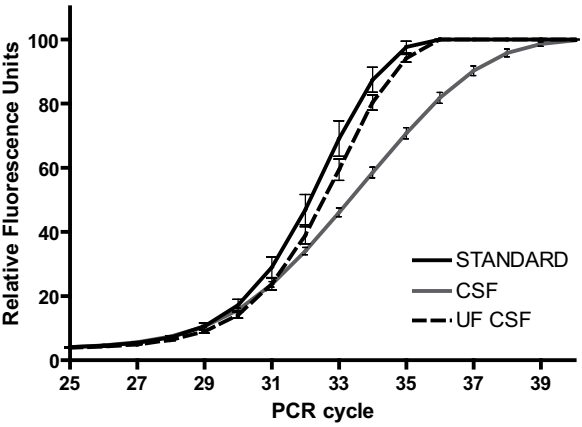
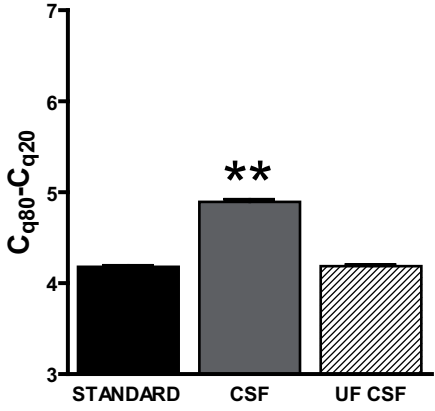
17
9
C-C1
C-LAβ
C-AD
C-FTLD
mtDNA-73
B
CSF Mitochondrial DNA (copies/ul)
200
160
120
80
40
0
29
15
**
4
3/3
4/3
4/4
APOE
mtDNA-73


A
mtDNA copies/ 18SrRNA
14 DIV
6
5
4
3
2
1
0
**
*
WT
APP/PS1
WT
APP/PS1
mtDNA-115
mtDNA-699
B
14 DIV
21 DIV
WT
APP/PS1
WT
APP/PS1
120KD
PSD95
50KD
Actin
50KD
Syn
50KD
Actin
C
PSD95/Actin
(% WT)
120
100
80
60
40
20
0
**
WT
APP/PS1
WT
APP/PS1
14 DIV
21 DIV
D
Synaptophysin/Actin
(% WT)
120
100
80
60
40
20
0
**
WT
APP/PS1
WT
APP/PS1
14 DIV
21 DIV

A

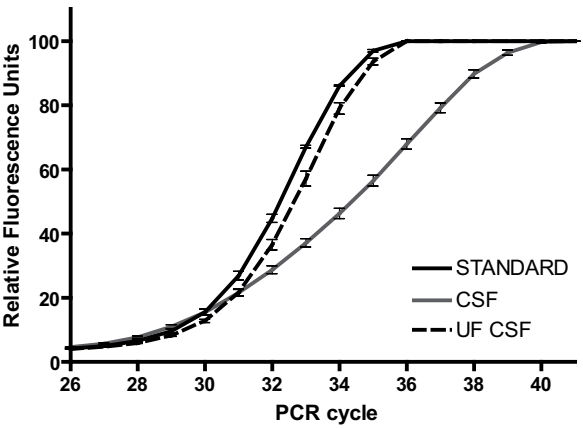
mtDNA-85

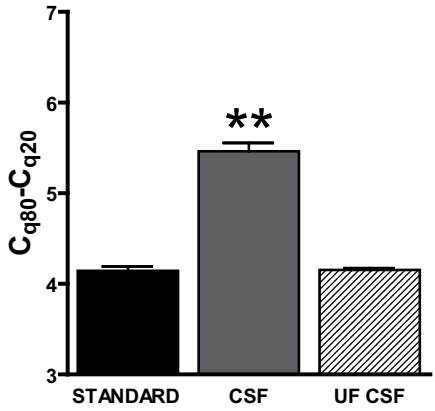




B

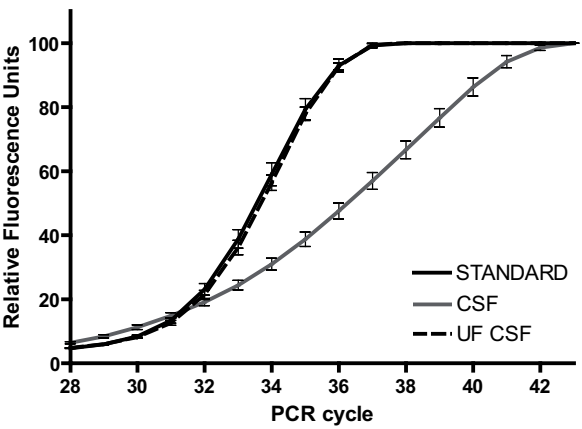
mtDNA-153

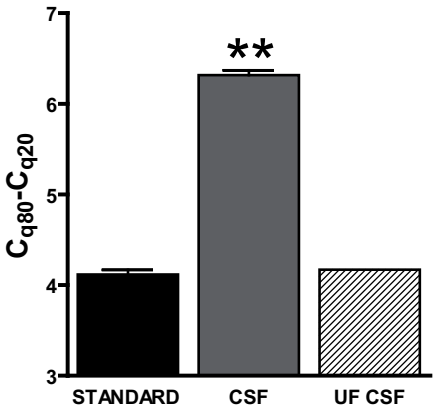


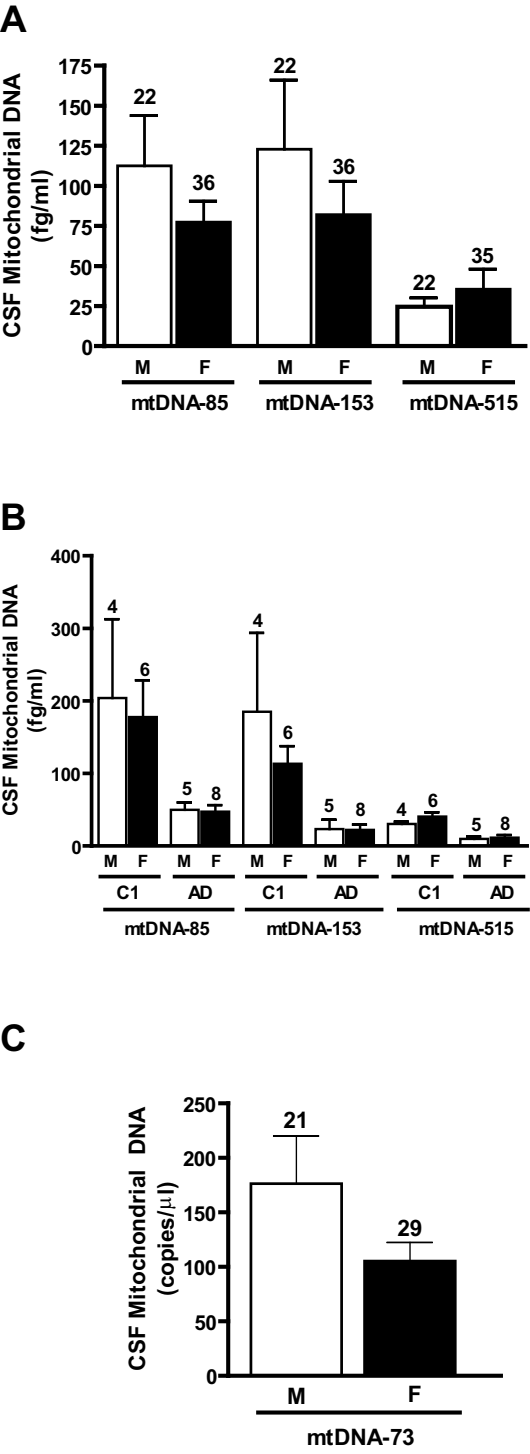


C

mtDNA-515






A
CSF Mitochondrial DNA (fg/ml)
175
150
125
100
75
50
25
0
22
36
22
36
22
35
M
F
M
F
M
F
mtDNA-85
mtDNA-153
mtDNA-515
B
CSF Mitochondrial DNA (fg/ml)
400
300
200
100
0
4
6
5
8
4
6
5
8
4
6
5
8
M
F
C1
AD
mtDNA-85
mtDNA-153
mtDNA-515
C
CSF Mitochondrial DNA (copies/μl)
250
200
150
100
50
0
21
29
M
F
mtDNA-73